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14. ABSTRACT
Toxicity is a major impediment to effective radiation therapy of locally advanced prostate cancer. Work under this award focuses on the potential of a novel class of pharmacological 'radiation protectors' (cysteine modifying agetns (CMAs) to reduce normal tissue toxicity of radiation therapy. During the third year of this award we continued to characterize effects of (the synthetic triterpenoid RTA 408 and the sesquiterpene lactone DMAPT that had emerged as a robust and selective radiation protectors of normal tissues. While radioprotective for normal tissues, both compounds also showed anti-tumor activity against four human prostate cancer cell lines grown as xenotransplants in mice. During the last year we have gained insights into the mechanism(s) of action underlying the opposite and beneficial effects of CMAs on normal and malignant tissues. A major effort focused on the effects these drugs on myeloid (bone marrow-derived) cells. This is based on our finding that inhibiting recruitment of myeloid cells obviates radiation protection of normal tissues by RTA 408. Others described that effects on myeloid cells also underlie tumor growth inhibition by synthetic triterpenoids. This circumstance raises the interesting perspective of a potential 'convergent' phenotype in myeloid cells elicited by RTA 408 and related compounds. During the last funding period we carried out phenotypic and proteomic analyses to define how RTA408 and related compounds affect myeloid cell phenotypes. This work has revealed novel leads and pathways relevant to cytoprotection. These will be pursued to improve development of this class of drugs for the radioprotection indication and as anti-tumor agents.

15. SUBJECT TERMS
Radiotherapy, Symptom Management, Signal Transduction, Drug Development

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INTRODUCTION:

Radiation therapy (RT) remains a key therapeutic option for prostate cancer, either alone or in combination with hormone therapy. However, the radiation dose that can be safely administered is often lower than the dose considered to be optimal to eradicate tumor cells in the vicinity of the primary lesion, for example pelvic lymph nodes. This is due, in large part, to 'collateral damage' by radiation, i.e. toxicity to the intestine and the bladder. Treatment strategies to escalate the dose of radiation to the pelvic sentinel lymph nodes are limited by normal tissue dose constraints that can't be surmounted by IMRT or particle therapy. Hence protection of normal tissue will be a critical requirement for future dose escalation trials in patients with locally advanced disease.

Existing radiation protectors including amifostine (1), sucralfate (2) and mesalazine (3) are of limited utility in selectively protecting the small and large intestines against radiation effects. This motivated us to explore under this award <u>the potential of a novel class of pharmacological 'radiation protectors' to reduce normal tissue toxicity associated with radiation therapy</u>.

We focused on exploring the potential of pharmacological inhibitors of NF-κB activity and glycogen synthase kinase(GSK)3 to provide radiation protection to normal gastrointestinal tissues. NF-κB inhibitors were chosen based on the known anti-inflammatory effects of the agents and GSK3 targeting agents were selected because they mimic select pro-survival effects of the PI-3-kinase/AKT pathway. Of note, some of the inhibitors tested (e.g. ethyl pyruvate, CDDO variants) also exert antioxidant activity.

<u>**Table 1:**</u> **Compounds under investigation.** The compounds indicated below were selected due to their radioprotective properties in zebrafish screens and in mice. All compounds used in zebrafish except RTA 408/CDDO were from Calbiochem/EMD. RTA 408 and other CDDO derivatives were provided by REATA Pharmaceuticals. Note that, in a previous progress report RTA 408 was referred to as TX425. Protection was achieved in zebrafish and mice at roughly equimolar concentrations where data are available in both model systems. Radioprotection of the GI system in zebrafish was selectively tested and observed for EP and CDDO as well as for LiCl, SB216763 and Azakenpaullone. Data were compiled from the following references (4-6). Zebrafish GSK3 inhibitor data from our laboratory are unpublished.

Pathway	Compound	Target(s)	Effective dose (in vitro)
NF-κB	Ethyl Pyruvate (EP)	NF-κBp65	1 mM
	RTA 408 (CDDO)	IKKβ/KEAP1-Nrf2	0.5 μM
GSK3	Lithium Chloride (LiCl)	GS3 (allosteric)	20 mM
	SB216763	GSK3 (ATP competitive)	5 μM
	Azakenpaullone	GSK3 (ATP competitive)	0.3 μM

BODY:

Work detailed in the preceding two annual reports led us to focus on a derivative of the synthetic triterpenoid CDDO (RTA 408). This was done as, among the agents listed in Table 1, RTA 408 appeared to be most effective and comparable to amifostine as it relates to radiation protection of the GI tract. Previously described work further demonstrated that RTA 408 radiation protection was selective, i.e. it did not extend to malignant tissues including prostate cancer xenografts and that radiation protection provided by RTA 408 requires recruitment of myeloid cells into irradiated tissues. Some of these results have since been published (7). In the last progress report we further presented data supporting the notion that the radioprotecive effect of RTA 408 is a 'class' effect of drugs that covalently modify the sulfhydryl groups of free cysteines on proteins (referred to ascysteine modifying agents (CMAs)); these include RTA 408 and other CDDO variants (8-13), ethyl pyruvate (4, 5) and the parthenolide derivative DMAPT (14).

We reasoned that elucidating the mechanism of action by which RTA 408 provides radiation protection to the GI system is likely to inform about and provide a reference point for the larger class of agents as well. In the following we will describe three independent lines of investigation providing new insights into and understanding of molecular mechanisms of radiation protection by CMAs:

1) Radiation protection by RTA 408 extends beyond the epithelial stem cell niche and encompasses stem cells of the hematopoietic system in the bone marrow;

2) RTA 408 also protects liver cells against genotoxic stress by DNA damaging agents by a paracrine mechanisms involving myeloid cells;

3) Proteomic analyses of myeloid cells illuminate additional target proteins in myeloid cells of potential relevance to cytoprotection.

1. Radiation protection by RTA 408 extends to the hematopoietic system in the bone marrow.

In colaboration with Dr. William H. Fleming (Oregon Stem Cell Center; OSHU) we detemined that RTA 408 increased survival of hematopoietic raidation syndroma associated with complete rescue of functionally competent hematopoietic stem cells. Specifically, the administration of a brief course of RTA 408 treatment beginning 24 h after bone marrow lethal doses of radiation significantly increased overall survival. Importantly, treatment with RTA 408 led to the full recovery of steady state hematopoiesis with normalization of the frequency of hematopoietic stem and progenitor cells. Moreover, hematopoietic stem cells from RTA 408-mitigated mice showed lineage-balanced, long-term, multilineage potential as determined by serial bone marrow transplantation, indicative of their normal self-renewal activity. The potency of RTA 408 in mitigating radiation-induced bone marrow suppression makes it an attractive candidate for potential clinical use in treating both therapy-related and unanticipated radiation exposure. In addition to support by this award, the results of this study were supported, in part, through the pilot project mechanisms of a CMCR award by an NIAID and have now been published (15).

2. Cytoprotection by RTA 408 extends to liver tissue damaged by the genotoxic agent DEN.

Based on the results obtained in the radiation setting and in collaboration with Dr. Jacob Rachmilewitz at Hadassah University in Israel we asked the question whether cytoprotection by RTA 408 extended beyond the radiation paradigm and encompassed other forms of genotoxic stress. Dr. Rachmilewitz focuses on repair of diethylnitrosamine (DEN)-induced DNA damager in the liver. In previous work he observed that myeloid cells (macrophages) recruited into injured livers improve DNA damage repair by secreting cytokines (16). Specifically, his data support a role of macrophage-derived heparin-binding epidermal growth factor (HB-EGF) in improved DNA repair; HB-EGF is an activating ligand of the EGFR receptor expressed by liver parenchymal cells (and gastrointestinal epithelial cells). Furthermore, preliminary unpublished work supports the hypothesis that inflammatory cytokines reduce the beneficial effect of HB-EGF on the DNA damage response. In collaboration with Dr. Rachmilewitz we determined that the RTA 408 variant CDDO-Me (RTA 405) accelerates dNA repair in DEN livers (Fig. 1) and that CDDO-Me serves to both, induce HB-EGF production (Fig. 2) and reduce secretion of TNFα and IL-1β (Fig. 3) by macrophages. Note that this work was accomplished under separate funding but relied heavily on the data accrued under this award (PC111002)). Several grant applications are pending to follow up on the work already accomplished and further dissect cytoprotection by regulation of paracrine cytokines elaborated by myeloid cells. The significance of these findings are that one RTA 408 target (HB-EGF) in myeloid cells has a documented role in DNA repair and fits the model of paracrine mechanisms linking myeloid cells to parenchymal tissue protection and repair.

Figure 1: CDDO-Me enhances DNA damage resolution in DEN-treated mouse livers. Mice were treated with either vehicle or CDDO-Me and then injected with DEN. Mice were sacrificed 2 and 6 days after DEN injection. Representative images of γH2AX staining in liver sections (n=3 mice in each group); images courtesy of Dr. Rachmilewitz).

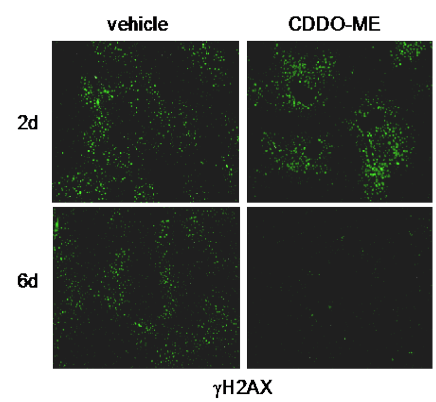


Figure 2: RTA 408 directly inhibits inflammasome-dependent IL-1β secretion and processing. Murine NG5 myeloid cells overexpressing NLRP3 exposed to LPS were treated with ATP for 45 min to induce NLRP3 activation. RTA 408 was added 15 min prior to ATP treatment. Results of immunoblot analysis of supernatants using an antibody detecting pro-IL-1β and mature IL-1β are shown. RTA 408 markedly reduces IL-1β secretion and processing in a dose-dependent fashion.

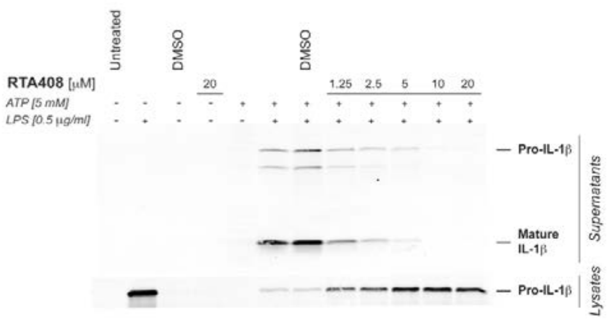
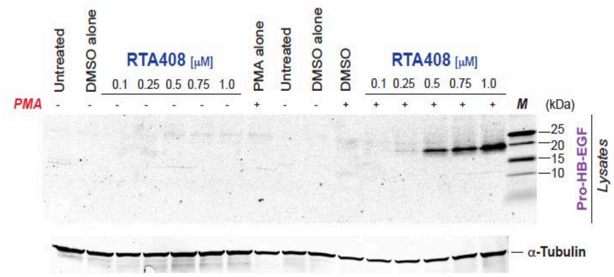


Figure 3: RTA 408 induces HB-EGF production by myeloid cells. Murine bone marrow-derived adherent cells were treated with IFNγ and LPS to induce an M1 macrophage phenotype. RTA 408 was added for 24 h in the presence or absence of PMA as shown. Results of immunoblot analysis of supernatants using an antibody detecting murine HB-EGF are shown. RTA 408 markedly enhanced HB-EGF expression in a dose-dependent fashion.



3. Novel target identification by proteomic analyses of myeloid cells

In collaboration with Dr. Emily Chen (Director, Proteomics Core, Columbia University, New York, NY) we have begun to monitor the effects of CMAs and, in particular RTA 408 on protein expression and phosphorylation in myeloid cells. We have completed a series of experiment in which bone marrow-derived myeloid cells (CD11b+) were subjected to cytokines inducing polarization into M1 (LPS, IFNγ) and M2 (IL4) phenotypes (Fig. 4). Controls in this experiment were cells untreated with polarizing cytokines and cells treated with a mixture of LPS/IFNγ/IL4 (M1/M2). This experiment was done in the presence and absence of RTA 408 and the experiment was repeated six times. Protein extracts from the treated cells were subjected to mass spectrometric analysis and protein abundance was tabulated. Treatment with RTA 408b induced marked changes in the protein profiles of myeloid cells. Importantly, these changes were highly dependent on the differentiation state of the cells. Specifically, M1 and M2 polarized cells were markedly different (Fig. 5) Differences were observed in NFkB target genes and proteins, antioxidant defense enzymes, DNA repair proteins and mitochondrial proteins. In addition, changes in the phosphoproteome were assessed. These data are currently being analyzed using advanced bioinformatics approaches. Perhaps the most striking finding is that RTA 408 has multiple effects on proteins controlling cell metabolism. We are currently performing functional tests on predictions emanating from the protein patterns as they relate to energy metabolism and mitochondrial function. This work promises to provide a novel and unprecendented view into the molecular mechanisms underlying cytoprotection by CMAs including but not restricted to CDDO variants.

Figure 4: Experimental design to identify molecular targets and patterns of CMAs in myeloid cells. Bone marrow-derived CD11+ myeloid cells were expanded in vitro for 7 days and then subjected to cytokine treatments in the presence and absence of RTA 408 (experiment with DMAPT pending). Samples were analyzed by Dr. Chen using high-resolution mass spectrometry. Further analyses are ongoing.

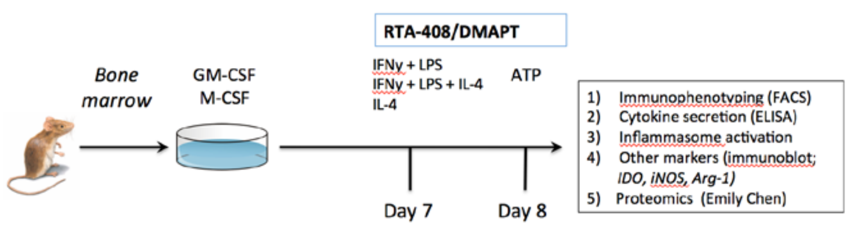
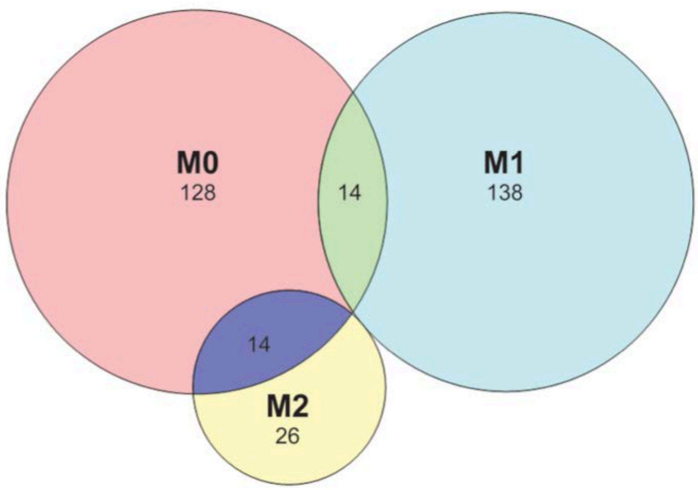


Figure 5: RTA 408 treatment alters protein abundance in myeloid cells. Results showing the most significant changes in macrophages polarized towards M1, M2 phenotypes or non-polarized are represented. The protein spectra are markedly different dependent on differentiation state.



KEY RESEARCH ACCOMPLISHMENTS:

- Identified radiation protection of different organ systems (GI tract, skin and hematopoiesis) by RTA 408.
- Identified a novel mechanism by which RTA 408 enhances cytoprotection through myeloid cells based on paracrine HB-EGF production.
- Performed unbiased proteomic screens to identify molecular targets and pathways in myeloid cells relevant to radiation protection; analysis of data ongoing.
- Secured additional support by the DoD focusing on preventing radiation damage in the skin of irradiated rats by CMA treatment.

REPORTABLE OUTCOMES:

- Two manuscripts published on selective radiation protection of gastrointestinal epithelium and the hematopoietic system by RTA -408.
- Two manuscripts on effects of RTA 408 on myeloid cell phenotypes are in preparation.
- One follow-up DoD award on radioprotection by CMAs has been awarded (PR141913 Luginbuhl/Rodeck Co-PIs).

CONCLUSION:

We have established that the cysteine-modifying compound RTA 408 is a robust radiation protector of multiple cell types and organ systems (hematopoietic, skin and gastrointestinal) in mice. Multiple lines of evidence spanning several organ systems point to a previously unrecognized mechanism of action of radiation protectors that depends on reprogramming myeloid cells. A concerted effort is under way to define the relevant molecular targets of RTA 408 and related compounds in myeloid cells; this is imperative as a corollary to further drug development and has already yielded interesting leads. This investigation will be continued and is the focus of several pending grant applications. In summary, the work performed under this award during the last 3 years has provided unique insights into radiation protection mechanisms amenable to pharmacological intervention. Our results support the hypothesis that systemic use of CMAs may optimally mobilize and skew myeloid cells with reparative properties.

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